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STUDIES ON SMALL COLONY VARIANTS OF STAPHYLOCOCCUS AUREUS

A PART OF A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF HYGIENE AND BACTERIOLOGY

1934

By
EDITH LOUISE SWINGLE

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STUDIES ON SMALL COLONY VARIANTS OF STAPHYLOCOCCUS AUREUS

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Small colony variants, or G forms, of bacteria have attracted considerable attention among students of bacterial variation as a result of Hadley's work (1931). In addition to a description of these variants and of the conditions under which they could be obtained, he also advanced evidence of their filterability with the belief that they represented a stage in the life cycle of the organism.

While Hadley's publication has provided the stimulus for recent investigation of these forms, the occurrence of what were in all probability similar variants had been noted by others. Since this work has been reviewed by Edwards and by Koser and Dienst, it is unnecessary to detail it here. It is believed, however, that the outline presented in Table I may be useful as a summary of the occurrence of these forms as they have been observed to date.

In the present investigation small colony variants of *Staphylococcus aureus* were studied with respect to their occurrence, morphology, physiological and antigenic properties, and filterability.

The usual type of variation encountered among the staphylococci has been a change in chromogenesis, the derivation of "albus" variants from "aureus" strains. Anyone who has worked with cultures of *Staph. aureus* has undoubtedly observed this change frequently. Pinner and Voldrich (1932) compared such

[1] The writer is indebted to Dr. S. A. Koser for suggestions and helpful criticism in the preparation of this manuscript.

"albus" as well as "roseus" and "citreus" variant forms with the original strains from which they had arisen and found them to differ in biochemical properties and in virulence, the variants being relatively avirulent. They did not observe the occurrence of rough colonies nor of any tiny colonies in the strains they studied. Bigger, Boland and O'Meara (1927) described six variant strains of *Staph. aureus* which differed in three properties, namely color, texture (smoothness or roughness of the colony) and viscidity. Various types of rough colonies and a small colony or G form were obtained from a single strain of *Staph. aureus* by Hoffstadt and Youmans (1932). This G form was isolated from lithium chloride medium and from rabbits inoculated with a smooth strain of the staphylococcus. More recently the same authors (1934) have described several white variants and G forms from orange strains of staphylococci. The G forms described by these authors illustrate well the fact that small colony forms are not all alike, for they differ in many ways from the small colony strains described in this paper.

ORIGIN OF THE SMALL COLONY STRAINS

The first small colony strains to be isolated by us were obtained from an agar slant culture of *Staph. aureus* which had been kept in the ice-box for three months and subsequently at room temperature for three weeks. Examination of plates made from this culture revealed several minute, colorless colonies scattered among the larger orange colonies typical of *Staph. aureus*.

Experiments with varied environmental conditions made in order to determine factors which favored formation of small colony variants included aging on agar and in broth, daily transfers and aging cultures in lithium chloride broth, and aging in 0.5 per cent casein digest medium. Plates were streaked from these cultures at twenty-four and forty-eight hours and weekly for a period of four weeks.

So far as could be observed, the age of the culture is especially associated with the formation of small colony variants, although other influences may play a part. Tiny colonies were not obtained from very young cultures, except occasionally from broth contain-

TABLE 1

Occurrence of small colony variants

DATE	INVESTIGATOR	ORGANISMS	CONDITIONS
1910	Jacobsen	*Eberthella typhosa*	Blood culture on Conradi agar autoclaved 30 minutes. Normal on agar autoclaved 15 minutes
1911	Fromme	*Eberthella typhosa*	Typical when isolated. Small colonies on subsequent transfer on agar
1914	Eisenberg	*Eberthella typhosa*	Old blood-broth and blood-bile cultures
		Serratia marcescens	Peptone water (7 per cent) cultures. Cultures on media containing various dyes
1918	Baerthlein	*Eberthella typhosa*	Typhoid stools streaked on Conradi-Drigalski medium
		Vibrio cholerae	Cholera stools on hemoglobin-extract-alkali-soda-agar
1922	Fürth	*Salmonella aertrycke* *Salmonella Schottmülleri*	Old broth cultures
1930	Koser	*Salmonella aertrycke*	Old broth cultures
1931	Edwards	*Shigella equirulis*	Direct cultivation, 25 per cent post-mortem examinations. Also in typical stock cultures
1931	Hadley	*Shigella dysenteriae* and other cultures	Serial transfers in $LiCl_2$ broth. Serial transfers in pancreatin broth. Aging nutrient broth cultures. Bacteriophage filtrates by "serial plates"

TABLE 1—*Concluded*

DATE	INVESTIGATOR	ORGANISMS	CONDITIONS
1931	Kuhn and Sternberg	*Escherichia coli* (45 strains) *Vibrio Metchnikovii* *Salmonella aertrycke* or *S. enteritidis* (mouse typhoid) *Salmonella suipestifer* *Pasteurella suiseptica* *Corynebacterium diphtheriae* *Proteus vulgaris* Proteus X19 *Shigella dysenteriae* *Eberthella typhosa* *Spirillum volutans* *Bacillus anthracis* *Bacillus sp.* (pseudoanthrax) *Mycobacterium tuberculosis*	Nutrient broth containing ammonia. Brief exposure to dilute phenol
1932	Hoffstadt and Youmans	*Staphylococcus aureus*	Serial transfers in $LiCl_2$ broth. Intravenous inoculation of rabbits
1933	Novak and Henrici	Pleomorphic organism between staphylococci and actinomycetes	Old broth cultures (7 weeks)
1933	Rettger and Gillespie	*Bacillus megatherium*	Serial transfers in $LiCl_2$ broth at 22°C.
1934	Koser and Dienst	*Shigella paradysenteriae* var. Sonne	Aging casein digest cultures. Old agar slants
1934	Raney and Kopeloff	*Lactobacillus acidophilus*	Old broth cultures
1934	Roe	*Clostridium Welchii*	Aging colonies and broth cultures
1934	Roos, Reichel and Clark	Hemolytic streptococcus	Brain broth containing 5 per cent normal rabbit blood

ing lithium chloride. They appeared after the cultures had aged for a week or two and were most numerous after three or four weeks. Lithium chloride had little effect in stimulating this

type of variation. Continuous transfer failed to bring out any large number of the small colony forms, and the few that appeared occurred irregularly.

In the cultures studied tiny colonies never occurred in large numbers, the most numerous being in the ratio of one small to sixteen large colonies. Usually there was only one small colony to 100 or more large colonies, and many times no minute colonies appeared. These small colony variants were unstable for the most part. Often the small colonies originally seen reverted wholly to the normal[2] form after two or three transfers on agar plates. Of several hundred colonies isolated, only nine remained small on repeated subculture over a period of several months. These nine were studied in the experiments reported in this paper.

A brief description of the origin of each variant strain follows.

V1, V2, V3: From an agar slant culture of *Staph. aureus*, strain M23, which was isolated originally from a furuncle. The slant had been incubated for 48 hours at 37°C. and then stored in the ice box for approximately three months. It had then been kept at room temperature for three weeks. The culture was rather dry and somewhat translucent.

V4, V5: From an agar slant of *Staph. aureus*, strain 95, which had been obtained from a case of food poisoning. The culture had been kept at room temperature for three weeks, at which time several small colonies appeared on plates streaked from it.

V6: From the third tube in a series of daily transfers of the Norsenski strain of *Staph. aureus* in 0.25 per cent lithium chloride broth.

V7: From the seventh tube in the same series as the preceding.

V8: From a culture of strain A of *Staph. aureus* which had been aging in 0.25 per cent lithium chloride broth at room temperature for one month. Strain A had been isolated from a case of osteomyelitis.

V9: From a nutrient broth culture of strain A which had been at room temperature for one month.

V10: From an agar slant of *Staph. albus* which had been kept at room temperature for two months. The strain reverted to the normal form on the second transfer so that no further studies were carried out with it.

[2] The word "normal" is used in this paper as the most convenient term to designate the type of colony of *Staph. aureus* usually observed, which is round and smooth, with a diameter of 1 to 2 mm. when grown on nutrient agar. recognized that this may not necessarily be the only normal form.

V11: From a culture of strain 105 in 0.5 per cent casein digest broth, kept at 37°C. for three weeks. All of the strains isolated returned to normal after two or three transfers. Strain 105 was originally isolated from a case of food poisoning.

MORPHOLOGY AND COLONY FORM

Well-isolated colonies of *Staph. aureus* are usually from 1 to 2 mm. in diameter when grown on nutrient agar. Those of the small colony variants could scarcely be discerned without the aid of a microscope. In the various strains they ranged from 0.04 mm., the smallest ever observed, to 0.5 mm., but most frequently they were 0.1 mm. in diameter. The limits of variation were quite constant for each strain, except when the culture appeared to be returning to the normal, at which time all sizes of colonies occurred.

With the exception of one strain, colonies of the variants were all smooth, round and convex, with entire margins. Strain V1, however, usually produced flat, rough colonies with slightly irregular margins. These variants had lost the power of pigment production.

Morphologically the variants were not strikingly different from the original strains, cells of which were about 0.8 micron in diameter, gram-positive, and arranged in irregular clusters. The cells of the different variants showed some individual differences and will be described separately. Several were similar to the normal form, gram-positive and grouped in small clusters or pairs. They were perhaps a trifle smaller than the usual form. Strain V3 showed a tendency to be gram-negative.

In variants V1 and V2 the cells were very irregular in size. Some were in tetrads, others in pairs or single. Within each group of cells there were marked contrasts in size. A tetrad might consist of one very large, balloon-like cell and three small ones, or two large and two small ones. When large cells occurred they were usually oval, about 1.8 by 0.9 microns, while the small ones were 0.5 micron in diameter.

Strains V4 and V5 also showed some variation in morphological characteristics. The organisms were in tetrads or pairs

and were not always spherical. The larger ones were about 1 by 1.2 microns and the smaller about 0.8 micron.

REVERSION OF VARIANT TO NORMAL FORM

Aging in nutrient broth. In order to study the return of the small colony to the normal form, the strains were inoculated into nutrient broth of pH 7.0, and incubated at 37°C. The development of these cultures was followed by streaking agar plates at intervals and observing the appearance of the colonies after forty-eight hours' incubation.

In the first series most of the strains were found to have a long lag phase. All of the tubes except one failed to show clouding at the end of twenty-four hours, although plates streaked at this time with a 1 mm. loop showed from 1 to 50 colonies. In forty-eight hours there was still no turbidity in any of the cultures. Only one culture, V1, showed clouding at the start and numerous colonies each time it was streaked on a plate. At the end of six days there was some clouding in all the tubes, and in some strains colonies of normal size (1 to 2 mm.) appeared along with the small colonies on plates.

Considerable difference was shown between the strains in their behavior in broth and in the rate at which they returned to the normal. These differences were apparent even in sub-strains which came originally from the same colony, notably V3a and V3b. V3a produced very minute colonies, 0.07 to 0.14 mm., at the end of forty-eight hours. After six days they were about 0.3 mm., and 60 per cent were 1 mm. in diameter after two weeks. After four weeks' aging in broth the colonies were all large. Strain V3b, on the other hand, never gave rise to a colony larger than 0.25 mm. In contrast to these is the behavior of strain V6 which showed 50 per cent normal colonies on plates streaked from twenty-four-hour broth cultures.

Spontaneous appearance of normal form. Reversion to the normal frequently occurred spontaneously. Sometimes in transferring from a stock culture, one or two large colonies with deep golden pigment appeared on the new slant and stood out sharply against the background of very small colonies which were scarcely

visible. Also in streaking plates from stock cultures of variants several large orange colonies occasionally appeared scattered among the more numerous tiny ones. This sudden reversion from variant to normal is quite different from the very gradual change described by Hadley for the G form of the Shiga dysentery bacillus and by Koser and Dienst for the Sonne dysentery bacillus. It is apparently due to changes taking place in an occasional cell but not affecting the culture as a whole.

Effect of glucose. This sugar had a marked effect on reversion as several strains showed almost 100 per cent normal colonies after forty-eight hours in glucose broth. All of the variants were streaked on plates of nutrient agar, veal-infusion agar, nutrient agar containing 0.5 per cent glucose, and veal-infusion agar to which 0.5 per cent glucose had been added, incubated under the same conditions and examined after forty-eight hours' growth.

Differences in behavior on these media were quite apparent. The colonies on nutrient agar were very small and only two strains formed any normal colonies. On veal-infusion agar the colonies were all larger, and two strains showed numerous normal colonies. Glucose added to nutrient agar also seemed to favor growth, as the colonies on this medium were distinctly larger than those on agar without glucose. The combination of glucose and veal infusion had the most marked effect. All of the strains which had ever reverted to the normal formed at least a few normal colonies on glucose veal-infusion agar. To illustrate these differences, the average colony measurements of one strain on the various media are given. The colonies of V2 were exceedingly small, 0.04 to 0.07 mm., on nutrient agar. On veal-infusion agar they ranged from 0.07 to 0.2 mm. On glucose agar they were 0.25 to 0.5 mm., with a few tiny colonies scattered on the plate, but on glucose veal-infusion agar they all looked like normal staphylococcus colonies, 1.0 to 1.8 mm. and of a deep orange color. Such studies indicate that the production of these small colony variants was linked with some form of altered metabolism since a change in the medium had such a profound effect on the variants.

Failure to obtain reversion. Three strains resisted all attempts

to make them produce normal colonies. The colonies of strain V1, which were small, flat and rough, never attained a size of more than 0.5 mm. in diameter. In addition to aging in broth, serial transfers were carried out in broth and on agar plates, the largest colonies being selected for transfer. Only small flat colonies continued to develop. Colonies on blood agar were even smaller than on nutrient agar. Strains V4 and V5 also failed to return to the normal. They showed no change after repeated transfers in broth and on agar slants. On blood agar, however, they developed larger orange colonies quite uniform in size and at least 1.0 mm. in diameter. These colonies did not represent a permanent return of the variant to the normal, for when transferred from blood agar to nutrient agar only very tiny colonies appeared. Since these strains failed to return to the normal, there is the possibility that they were contaminants rather than variants of the original staphylococcus. However, since their cultural and morphological characteristics on blood agar resembled so closely those of the original cultures, it is believed that they really represent variants which did not return to the normal on a standard medium within the period of observation.

In this group of small colony strains the cultures exhibited all degrees of stability, ranging from those which continually gave a few large colonies on each subculture to those which never reverted wholly to the normal.

INFLUENCE OF MEDIUM UPON GROWTH

Effect of blood. In addition to complete reversion to the normal a temporary stimulation of growth was found to occur under certain conditions. After twenty-four hours' growth on blood agar the colonies were all small, appearing much as they did on nutrient agar. At the end of forty-eight hours the colonies of two strains had become 1 mm. in diameter and had acquired a pale orange color. They looked like typical staphylococcus colonies, but differed from those of the parent strain in that the colonies were smaller and not hemolytic. When transferred back to nutrient agar only tiny colonies developed. Although cultured repeatedly on blood agar, they did not become hemolytic nor did

they acquire permanently the property of forming large colonies when cultured again on nutrient agar. None of the other variants showed this sudden development of all the colonies to a large size.

Effect of other organisms. In examining some old nutrient agar plates of strain V4 upon which two contaminants had appeared, it was observed that around the two stray colonies the colonies of the variant had become quite large and slightly pigmented. This appearance was confined to the immediate vicinity of the contaminating colony. The effect was similar to the "satellite phenomenon" shown by mixed cultures of *Staph. aureus* and *Hemophilus influenzae* on blood agar in which the *H. influenzae* grows much more luxuriantly in the neighborhood of a staphylococcus colony. The contaminants were large, non-motile, gram-positive, spore-forming rods.

These two organisms and also a laboratory stock culture of *Bacillus subtilis* were studied for their effect upon all of the small colony variants. Nutrient agar plates were streaked with cultures of the variants and a single streak of the other organism was made along the center of the plate at right angles to the other streaks. The plates were examined at the end of forty-eight hours at 37°C. and again at the end of ten days at room temperature. *B. subtilis* had no effect upon any of the small colony strains. The two contaminants affected two variants very markedly. Strains V4 and V5 grew much more luxuriantly near the center of the plate where the contaminant was streaked than at the edge of the plate. There was a gradual gradation in size from the larger colonies adjacent to the contaminant to those only slightly enlarged about 1 cm. away. Beyond that the colonies were uniformly small. The nature of the growth-promoting substance produced by these contaminants is unknown.

Effect of glucose and veal infusion. The influence of glucose in causing reversion of the variants to the normal form has been noted. It had also been observed in other experiments that colonies were sometimes larger on veal infusion than on nutrient agar, especially in the case of strains V4 and V5 whose growth has already been shown to be particularly subject to changes in the

composition of the medium. Strain V5 formed only very tiny colonies, 0.07 mm. to 0.13 mm., on nutrient agar. On veal infusion the colonies were 0.5 mm. to 0.6 mm., on glucose agar they were all about 0.2 mm., and on glucose veal-infusion agar they were 1.0 mm. across. However, these large colonies formed only tiny colonies when transferred again to nutrient agar.

FERMENTATION REACTIONS

The fermentation reactions of the small colony variants were compared with those of the original strains from which they were obtained and also with cultures which had been brought back from the small colony state to normal colony size by aging in broth or by other methods.

The cultures were inoculated into broth tubes containing 0.5 per cent of glucose, lactose, or sucrose, with brom thymol blue as an indicator. The reactions, as indicated by changes in the indicator, were observed at the end of twenty-four hours, forty-eight hours, one week, and two weeks, at which time plates were streaked.

The original staphylococcus strains, with one exception, attacked glucose, lactose, and sucrose, and produced a definite acid reaction in twenty-four hours. The variants differed in the time required to show any reaction and in the sugars which they were able to utilize. After twenty-four hours some of the tubes showed very slight clouding but there was no change in the indicator. At the end of forty-eight hours all of the glucose tubes showed abundant growth and a distinct acid reaction. Plates streaked from these cultures at times yielded only small colonies, while at other times both small and large colonies appeared. Four strains produced acid and formed only tiny colonies. One strain formed acid from glucose and the plates yielded mostly tiny colonies with a very few normal ones, while another fermented glucose and showed normal and small colonies in about equal numbers. When some reversion from the variant to normal had occurred by the time acid production was first evident, it was difficult to decide whether the carbohydrate had been attacked by the variant, by the normal form, or by both. How-

ever, since fermentation of glucose did occur in some tubes in which there was no reversion, or only slight reversion, of the culture to the normal, it may be presumed that the small colony variants did attack glucose, although somewhat more slowly than the normal strains.

Lactose not only was not fermented by the small colony strains but seemed to be inhibitory to their growth. Most of the tubes showed no clouding at the end of twenty-four hours' incubation and few or no colonies appeared on plates streaked from them. After forty-eight hours some of the tubes still showed no clouding and only scant growth on streaked plates. In a week there was moderate clouding in most of the tubes, but a few strains failed to grow. The acidity of the medium never became greater than pH 6.8.

The reactions in sucrose were similar to those in lactose. There was usually no clouding or change in reaction in twenty-four hours and only slight clouding in forty-eight hours. Two strains, V5 and V6, produced an acidity of pH 6.3 at the end of a week. The former had reverted almost completely to the normal at the time that this acidity was first observed.

The following table (Table 2) presents some of the reactions produced by these variants in glucose, lactose, and sucrose broths. It illustrates the feeble fermentative capacity of the small colony variants. In contrast, all of the strains which had been brought back to the normal fermented glucose, lactose and sucrose as readily as did the original normal strains.

BEHAVIOR OF VARIANTS UNDER ANAEROBIC CONDITIONS

In order to compare the growth of both the variant and normal strains under aerobic and anaerobic conditions, nutrient agar plates were streaked in duplicate from each culture. One set of plates was placed in a vacuum desiccator, evacuated until the pressure in the jar was reduced by 700 mm. of mercury and any remaining oxygen absorbed with pyrogallic acid and sodium carbonate. The other set of plates was kept under aerobic conditions. Both aerobic and anaerobic plates were incubated at 37°C. for forty-eight hours.

The normal cultures grew luxuriantly and produced abundant

pigment in the presence of oxygen, while under anaerobic conditions the colonies were slightly smaller in size and showed no

TABLE 2

Fermentation reactions of small colony variants

STRAIN	CLOUDING	REACTION	COLONIES
		Glucose*	
V1	Moderate	6.5	Many, all small
V2	Abundant	6.3	50 per cent normal; 50 per cent small
V3a	Moderate	6.5	Few, all small
V3b	Abundant	6.3	Many, all small
V5	Abundant	6.3	Many, all small
V6	Abundant	6.0	33 per cent normal; 67 per cent small
V7	Abundant	6.0	33 per cent normal; 67 per cent small
V8	Abundant	6.0	Many small; few 0.2 mm.
V9	Slight	6.7	Few, small
		Lactose	
V1	Abundant	6.8	Many, small
V2	None	6.9	None
V3a	None	6.9	Very few, small
V3b	None	6.9	None
V5	Moderate	6.9	Many, all small
V6	Moderate	6.9	98 per cent normal; 2 per cent small
V7	Moderate	6.9	98 per cent normal; 2 per cent small
V8	None	6.9	None
V9	None	6.9	Many, all small
		Sucrose	
V1	None	7.1	None
V2	None	7.1	None
V3a	None	7.1	2 colonies, small
V3b	None	7.1	None
V5	Moderate	6.9	Few, all small
V6	Abundant	6.9	98 per cent normal; 2 per cent small
V7	None	7.1	None
V8	None	7.1	None
V9	None	7.1	None

* These are the reactions after 48 hours' incubation. The reactions in lactose and sucrose showed no change after 7 days' incubation.

pigment. Most of the variants grew better aerobically than anaerobically as indicated by the number and size of the colonies.

Usually there were distinctly fewer colonies on the anaerobic than on the aerobic plates. The colonies which developed in the absence of oxygen were often extremely minute. Thus the aerobic colonies of strain V5 were about 0.1 to 0.13 mm., while the anaerobic colonies were less than 0.01 mm. in diameter. Anaerobic conditions seemed to inhibit reversion to the normal form in those strains which had a tendency to revert under the usual aerobic conditions.

FILTRATION EXPERIMENTS

The question of filterability of small colony variants is of especial interest in view of the controversy as to whether bacteria possess a filter-passing stage in their life history. Although the morphology of these small colony variants of staphylococci would suggest the impossibility of their passage through Berkefeld filters, small units representing a filter passing stage might be associated with the large cells. Tests for filterability were made with both normal and variant strains.

Preparation of cultures. Kendall's K medium (1931) was used in the first series of filtrations. Cultures were inoculated into 150 cc. amounts of the medium and samples were withdrawn for filtration after forty-eight hours and at weekly intervals for one month. Cultures were also subjected to daily transplants in the K medium and filtered at the end of the third, seventh and twelfth transfers. Plates were streaked from each culture before filtration in order to determine whether any visible changes in the colony form had occurred.

Cultures in nutrient broth of pH 7.4 were also used. Samples were withdrawn for filtration at the end of forty-eight hours, one week and four weeks. Plates were streaked at the time of each filtration in order to determine the proportion of cells in the small colony state. Various degrees of reversion were revealed by these plates from only tiny colonies at the end of forty-eight hours to 90 per cent normal colonies after a month.

Technique of filtration. Berkefeld N filters shown to be impervious to a twenty-four-hour-broth culture of *Serratia marcescens*

were used. Usually about 15 cc. of the culture was filtered. The time required for the filtration was short, usually about five minutes. The filtrates were collected in test tubes within the suction flasks.

Tests of filtrates. Each filtrate in amounts of 1.0 cc. and 0.25 cc. was inoculated into 10 cc. tubes of glucose veal-infusion broth. K medium was used, in addition, for all of the K medium filtrates. The broth tubes were held at 37°C. for one month and examined at intervals for the appearance of any sign of growth. The Hauduroy technique of serial plate transfers was also used. Material was transferred from plate to plate at intervals of forty-eight hours for 6 transfers, the plates being examined microscopically each time before a fresh transfer was made. The remainder of each filtrate was kept at room temperature for one month, examined for growth and a few drops spread on an agar plate.

Results of filtration tests. Although a total of thirty filtrations was made, growth could not be detected in any of the media inoculated with filtrates from variant or from normal cultures. The broth tubes and the tubes of filtrate allowed to stand at room temperature occasionally developed a slight cloudiness, but stains made from these tubes showed nothing except a slight precipitate or bits of débris. Plates heavily inoculated with material from these cloudy tubes showed no growth.

As the dried broth accumulated on the plates used for the Hauduroy transfer technic after about the third or fourth transfer it formed a thin film, but stains made of this film showed nothing that might be interpreted as being a bacterial cell. In subsequent transfers discrete colonies could not be detected in the place of the film.

PATHOGENICITY

During the preparation of agglutinating sera it was observed that rabbits receiving intravenous inoculations of certain of the normal strains frequently became emaciated and sometimes died, while animals which received the variants did not develop unto-

ward symptoms. Such evidence suggested a loss of virulence of the small colony strains. The following experiments were carried out in order to verify this point.

Two series of cultures were selected. The first comprised normal strain A, a variant of this strain, V8, and a normal strain, V8-N, recovered from the variant; the second, strain 95 and its variant, V5. The organisms were grown on agar slants for twenty-four hours, suspended in saline, and standardized. Young rabbits weighing about 1500 grams each received intravenously one billion organisms of the strain under test. The animals were

TABLE 3

Effect of staphylococci upon rabbits

STRAIN	TYPE	LOSS IN WEIGHT	GROSS PATHOLOGY	SMEAR OF PUS FROM KIDNEY LESION
		grams		
A	Normal	300	Multiple abscesses in both kidneys	Gram-positive cocci
V8 (from A)	Small colony	None	None	No organisms
V8—N	Normal from variant	None	Pale area with tiny abscesses in one kidney	Gram-positive cocci
95	Normal	125	None	No organisms
V5 (from 95)	Small colony	Gain	None	No organisms

watched and changes in weight recorded for a week, after which they were killed and examined for gross lesions. Table 3 summarizes the changes observed.

The results with strain 95 and its variant are not conclusive because the normal organisms lacked sufficient virulence to produce macroscopic lesions in the rabbit although a loss of weight was observed. With strain A the loss in weight following injection, together with the renal abscesses seen at autopsy indicate a certain degree of virulence. In contrast to this is the complete lack of any detectable change in the rabbit which received an equal number of organisms of the variant strain. These results confirmed the previous observations that these variants are relatively avirulent. The behavior of the organism which had

regained its normal form from the variant condition was inconclusive. It did give rise to a few lesions in one kidney, but not to the extent observed with the original strain.

This decrease in virulence would lead one to believe that these particular variants are of slight importance in disease processes.

AGGLUTINATION

The original strains were not serologically specific, all showing some cross agglutination. Variants of 3 of the 4 normal strains studied were antigenically similar to their source strains as determined by agglutination reactions, and the same variants showed a like antigenic relationship to each other. The titers of the sera prepared against the parent strains were somewhat lower when tested with their variants. In the fourth instance the two variants from strain 95 showed no agglutination with 95 serum. These organisms behaved in a somewhat anomalous manner in other reactions and had never reverted to any permanent normal forms.

Sera prepared against the small colony strains likewise agglutinated the normal organisms, although the titers were rather low against both the variants and the normal bacteria. This was probably due to the fact that only killed bacteria were used in the process of immunization to prevent the possibility of any reversion to the normal taking place after inoculation. The following table illustrates the results obtained in agglutination tests.

The findings are at variance with the report of Hoffstadt, Youmans, and Clark (1934) that the G organisms of *Staph. aureus* were agglutinated only by G sera. They support the idea that this type of variation represents a change in physiological behavior rather than alteration in the actual composition of the organisms.

RATE OF MULTIPLICATION

The behavior of the small colony variants under the conditions in which they were studied pointed either to a very prolonged lag phase, a much retarded growth rate, or to a combination of both of these factors. A more accurate comparison of the rates of multiplication of normal and variant staphylococci was obtained

by following quantitatively the increase in the numbers of cells in broth cultures of the organisms. The normal strain, A, and variant, V8, were selected because V8 had proved to be quite stable, so that the results would not be confused by the reversion of numerous variant cells to normal. For further comparison a strain, V8-N, which had reverted from the variant to the normal form, was also used.

The organisms were grown on agar slants for twenty-four hour and suspensions in saline of approximately equal turbidity were

TABLE 4

Agglutination of various staphylococci with 95 (normal) serum

STRAIN	CONDITION	1:40	1:80	1:160	1:320	1:640	CONTROL
95	Normal	4+	4+	4+	3+	2+	–
V4	Variant from 95	–	–	–	–	–	–
V5	Variant from 95	–	–	–	–	–	–
Norsenski	Normal	4+	4+	2+	1+	1+	–
V7	Variant from Norsenski	2+	1+	1+	–	–	–
M23	Normal	4+	4+	4+	4+	3+	–
V1	Variant from M23	3+	3+	3+	3+	2+	–
V2	Variant from M23	4+	4+	3+	3+	3+	–
V3	Variant from M23	3+	2+	1+	–	–	–
A	Normal	4+	4+	4+	3+	2+	–
V8	Variant from A	4+	4+	4+	3+	2+	–

prepared. One 1 mm. loop of each suspension was inoculated into 100 cc. of broth. Plates were poured immediately to determine the number of viable organisms per cubic centimeter in each culture at the beginning of the experiment. Appropriate dilutions in sterile saline were made and plates poured in duplicate at the end of two, four, eight, twelve, twenty-four, and forty-eight hours. These plates were incubated for three days at 37°C. to insure the development of all colonies, especially the tiny ones, to a visible point. The numbers of colonies were counted and the numbers of viable organisms per cubic centimeter in the culture at each time of plating were estimated. From these figures the

progressive increase in the number of cells in the cultures could be calculated. Table 5 is typical of the results obtained.

The contrast between the rate of multiplication of the small colony variant and that of the normal strain is brought out sharply by the figures in the second part of the table which express approximately the ratio of the number of organisms to the original number at each time of plating. For the first two hours,

TABLE 5

Multiplication in nutrient broth

TIME	NORMAL STRAIN A	VARIANT V8	NORMAL RECOVERED FROM VARIANT V8–N
	Colonies per cubic centimeter		
hours			
0	2,850	2,080	2,120
2	3,800	4,530	3,340
4	31,300	5,870	17,300
8	8,500,000	19,600	22,900,000
12	113,000,000	33,300	79,000,000
24	370,000,000	960,000	520,000,000
48	790,000,000	113,000,000	1,010,000,000
	Ratio to original inoculation		
0	1	1	1
2	1.3	2	1.6
4	11	2.8	8.2
8	3,000	9.4	10,800
12	40,000	16	37,300
24	130,000	460	245,000
48	277,000	54,000	476,000

which probably represents the period of lag, the three cultures behaved very much alike, the increase in numbers being relatively slight. At the end of four hours, both normal strains were multiplying distinctly more rapidly than the variant. In twelve hours the number of viable cells per cubic centimeter in the variant culture was only sixteen times that of the original inoculum, while in the normal strain the number was 40,000 times that at the

beginning of the experiment. Similar differences are quite apparent in the figures for twenty-four hours and forty-eight hours. The strain V8-N, which had reverted to the normal, appeared to increase as rapidly as the original normal strain. The cultures were not followed after forty-eight hours because at this time several normal colonies appeared on plates streaked from the variant cultures. Such organisms, which presumably had recovered their more rapid rate of reproduction, would confuse further study of the slower growing variants.

DISCUSSION

All attempts to demonstrate the association of a filterable or "virus-like" stage with these organisms were unsuccessful. The results differ in this respect from those of Hoffstadt and Youmans (1932). The fact that these forms occur rarely and that staphylococcus cultures may be studied under diverse conditions without the appearance of small colony forms would cast considerable doubt upon the assumption that they constitute an essential stage in the life cycle of the organism.

The small colony variants are characterized by a change of physiological properties. The deviation from the familiar form which first brought them to the attention of investigators was a decrease in colony size. Further studies on these staphylococcus variants have revealed a rate of multiplication much slower than that in the normal form, a decrease in fermentative powers, loss of pigment production, loss of virulence, and often an increase in the size of the individual cells. These changes seem to be chiefly physiological. The fundamental variation is probably one of metabolic activity, since the use of enriched media accelerated reversion to the normal form.

It would appear, then, that as a result of age or of some chemical injury there is damage to an occasional cell which is retained through a number of successive cell generations. This does not affect equally all of the cells in a culture. In a new environment, such as is obtained by transfer to new media, an occasional cell may recover its lost characteristics. This return to the normal likewise does not affect equally all of the organisms in the culture.

The change from small to large colonies was usually abrupt and colonies of intermediate sizes seldom appeared. At no time was there a uniform increase in colony size of all the organisms in a culture. However, the proportion of such cells recovering could be increased by enrichment of the media in which they were grown.

SUMMARY

1. Several small colony variants of *Staphylococcus aureus* were isolated from old broth or agar cultures or from cultures in lithium chloride broth. Their occurrence was irregular and rare.

2. The colonies of these organisms were very small and lacked pigment. The cells were similar to those of the normal form in some cases, while in other strains there were marked irregularities in size.

3. The small colony forms were characterized by a change of physiological properties, such as loss of fermentative powers and a relatively slow rate of multiplication. Growth could be stimulated in some strains by the use of veal infusion and glucose, and in others by the presence of blood in the medium or of certain contaminants on the plates.

4. Reversion to the normal form occurred occasionally on agar. It could be enhanced by aging in broth or by enrichment of the media.

5. The variant organisms were avirulent for rabbits in the doses used.

6. The variant organisms could not be recovered from filtrates of Berkefeld N candles.

7. Agglutination tests showed most of the variants to be antigenically similar to their source strains.

8. It is suggested that these small colony forms occur as the result of injury to an occasional cell which is retained through successive cell generations.

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PLATE 1

FIG. 1. Cells of normal staphylococcus, M23, from twenty-four-hour colonies on nutrient agar.

FIG. 2. Cells of small colony variant, V2, from twenty-four-hour colonies on nutrient agar.

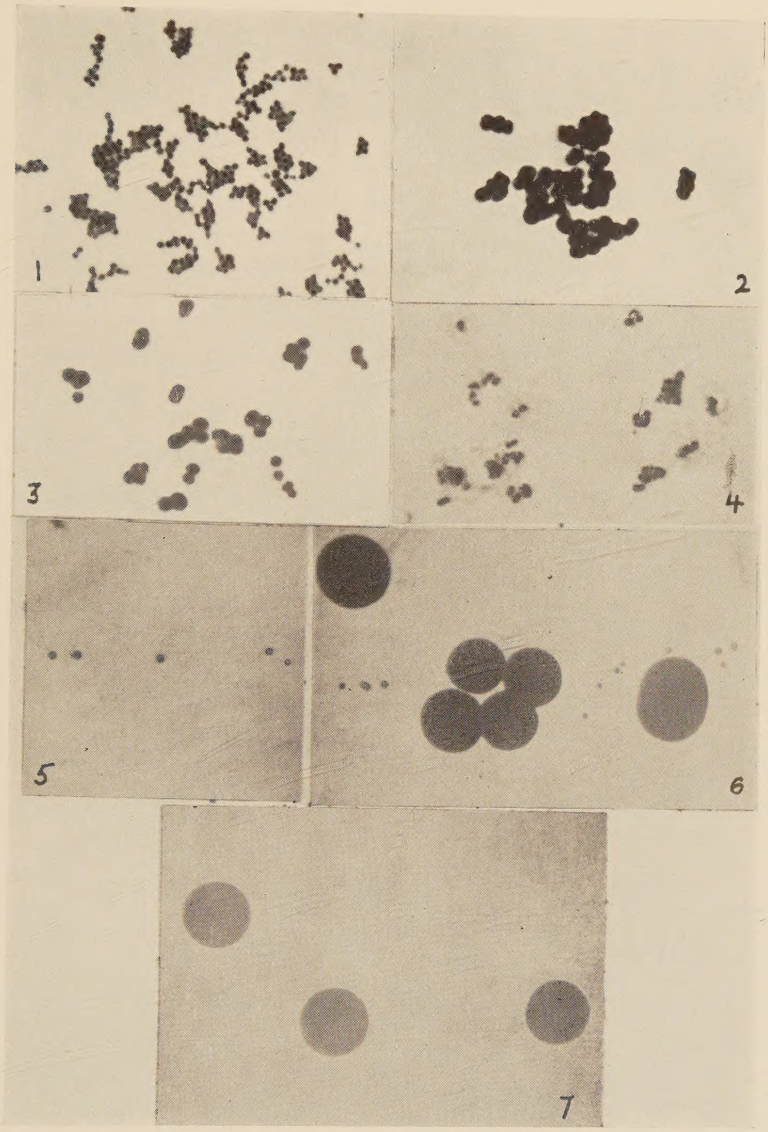
FIG. 3. Cells of small colony variant, V1, from twenty-four-hour colonies on nutrient agar.

FIG. 4. Cells of small colony variant, V5, from twenty-four-hour colonies on nutrient agar.

FIG. 5. Colonies from twenty-four-hour-broth culture of small colony variant, V2. × 10.

FIG. 6. Colonies from three-day-broth culture of V2 showing partial reversion to normal. × 10.

FIG. 7. Colonies from seven-day-broth culture of V2 showing complete reversion to normal. × 10.



(Edith L. Swingle: Variants of Staphylococcus Aureus)